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THE EFFECT OF ACUTE RENAL FAILURE ON

MUSCLE PROTEIN TURNOVER IN THE RAT AND

IMPLICATIONS FOR THERAPY

Annual/Final Report

Arthur S. Kunin, M.D.

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Acute renal failure (ARF) often accompanies and complicates the management of combat casualties suffering from extensive trauma and prolonger hypotension. Despite replacement of blood, fluids and electrolytes, expert surgical care, antibiotics and hemodialysis, the overall mortality has remained high (50-60%). The underlying cause of death is not renal failure now that dialysis is available, but the associated hypercatabolic state in which wound healing is delayed, increased susceptability to infection is present and nutritional inanition results. Negative muscle protein balance is a major feature in this metabolic

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setting and there is suggestive evidence that protein catabolism and mortality in acute renal gailure may be modified by therapy directed towards reducing muscle protein degradation.

This study was designed:
1. to explore, in a model of ARF in the rat, the balance of protein synthesis and degradation in a series of representative muscles: the diaphragm, heart, soleus and extensor digitorum longus.
2. to devise a therapeutic regimen to reduce muscle protein catabolism and enhance muscle protein synthesis.

A. Goals: 1. Develop a reproducibla model of ischemic acute renal failure in the young rat.
2. Determine the in vitro pattern of protein turnover in these muscles, following ARF and nutritional deprivation.
3. Conduct in vitro muscle incubation studies utilizing various nutritional substrates theoretically able or known to decrease muscle breakdown, in order to screen for efficacy.
4. Once promising agents are identified, to test their in vivo effectiveness by infusing the compound under evaluation intravenously in rats with ARF and appropriate controls and, following predetermined intervals of treatment, sacrifice the rats, isolate the muscles and incubate them in vitro to simultaneously assay them for muscle protein synthesis and degradation.

B. Methods:

Induction of ARF: accomplished by bilateral clamping of the renal pedicles for one hour.

Assay of muscle protein synthesis and breakdown: The isolated thin muscles are incubated in oxygenated, buffered Krebs-Ringer medium for 2 hours. The basic media contain glucose, ^{14}C-labeled phenylalanine. Muscle synthesis is assayed by determining the incorporation of ^{14}C-phenylalanine into the acid insoluable muscle fraction. Net protein degradation is assyaed by tyrosine loss from muscle into the incubation media. Protein breakdown is calculated as the algebraic sum of synthesis and net protein degradation.

C. Results:

In the first series of experiments in which ARF was induced, the rats allowed free access to water and no food, the pattern of muscle protein turnover was significantly negative. Body weights fell identically in both experimental and sham-operated controls from 0 thru 96 hours of study. Despite the addition of leucine to the media, protein synthesis fell in all muscles except the heart. The pattern of protein breakdown in these individual muscles was differentially altered both in ARF and in shams, findings obscured in whole body balance studies. Leucine added to the incubation media was most effective in heart stabilizing both protein synthesis and breakdown in ARF, suggesting selective therapeutic targeting of leucine in cardiac muscle.

When solutions of hypertonic glucose (23%) with or without insulin, or hypertonic glucose with either leucine or with a mixture of both essential or nonessential amino acids were infused intravenously in rats with ARF, the results on muscle protein turnover were better than when the animals were allowed only free access to water. Preliminary results however reveal that there were no differences in the efficacy of glucose vs glucose plus insulin or glucose plus leucine vs glucose plus multiple amino acids. We suggest on the basis of these preliminary studies in this model that there may be no special advantage to "super-therapeutic renal failure concoctions" over intravenous hypertonic glucose alone. If these findings are substantiated, it could mean simplification of treatment of patients with acute renal failure, simplification of supply problems for military medical units and a decrease in costs of intravenous solutions in acute renal failure.

Summary

Introduction:

Acute renal failure (ARF) often accompanies and complicates the management of combat causalties suffering from extensive trauma and prolonged hypotension. Despite replacement of blood, fluids and electrolytes, expert surgical care, antibiotics and hemodialysis the overall mortality has remained high (50-60%). The underlying cause of death is not renal failure now that dialysis is available but the associated hypercatabolic state in which wound healing is delayed, increased susceptability to infection is present and nutritional inanition results. Negative muscle protein balance is a major feature in this metabolic setting and there is suggestive evidence that protein catabolism and mortality in acute renal failure may be modified by therapy directed towards reducing muscle protein degradation.

This study was designed:
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Foreword

In conducting the research described in this report, the investigator adhered to
the "Guide for the Care and Use of Laboratory Animals," prepared by the
Committee on Care and Use of Laboratory Animals at the Institute of Laboratory
Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23,
Revised 1978).

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THE EFFECT OF ACUTE RENAL FAILURE ON MUSCLE PROTEIN TURNOVER IN THE RAT AND IMPLICATIONS FOR THERAPY

Body of Report

A. The Problem
1. Introduction:
Acute renal failure frequently complicates the management of combat casualties suffering from severe trauma and prolonged hypotension. Despite frequent hemodialysis and other supportive measures, the overall mortality averages 50-60%. The cause of death, now that dialysis is available, is not renal failure but the associated hypercatabolic state which delays wound healing and increases susceptibility to infection. Since negative muscle protein balance is a major feature in this metabolic setting and there is suggestive evidence that protein catabolism and mortality in acute renal failure may be modified by therapy, we plan to study muscle protein synthesis and degradation in a model of acute renal failure in the rat to systemically identify those factors influencing protein turnover and to devise a therapeutic approach which helps to reduce muscle catabolism and enhance recovery.

2. Objectives and Approach:
Using the young rat (in view of its genetic homogeneity, the requirement that thin muscles be utilized in the testing procedures, reduced experimental costs) it is planned to:

a. Develop a model of reproducible ischemic acute renal failure (ARF).

b. Determine the in vitro pattern of muscle protein turnover in specific representative muscles: the heart, diaphragm, extensor digitorum longus (EDL) and the soleus, following ARF and nutritional deprivation in experimental and in sham-operated control rats.

c. Conduct in vitro studies utilizing various nutritional substrates already known or theoretically able to decrease muscle protein degradation and/or stimulate muscle protein synthesis, in order to screen for their efficacy by incubating them with the various muscles isolated from both groups of rats.

d. Once promising agents are identified, to test their in vivo efficacy by infusing the compound under study intravenously into the cannulated jugular veins of rats with ARF and in appropriate controls. Following predetermined intervals of treatment, the rats will be sacrificed, the various muscles isolated and protein turnover studied.

B. Background
Two major scientific advances have significantly decreased mortality in

acute renal failure. The first, and perhaps the major advance, was the development of a rational approach to fluid and electrolyte therapy in patients with acute renal failure (1). The second was the development of dialysis methodology to sustain life when renal failure is prolonged. Despite the advances, mortality from acute renal failure in military casualties still averages 50-60% (2). The cause of death is not renal failure itself now that dialysis is available, but the associated toxic catabolic state that delays wound healing and leads to infection kand other systemic complications.

Studies by the Army Surgical Research Team reported in: Battle Casualties in Korea, Volume IV, Post-traumatic Renal Insufficiency (3,4) summarizes many of the important observations made on the clinical course, complications and therapy at the Renal Insufficiency Center at Wonju, Korea. To these have been added further observations from Vietnam by Donadio and Whelton (5) and those of Lorden and Burton from Clark USAF Hospital in the Philippines (2)

Teschan et al,in the Korean studies described the accelerated rate of azotemia and hyperkalemia resulting from the marked tissue catabolism characterizing post-traumatic renal insufficiency noting especially the early signs of weight loss and emaciation as well as edema formation on less than conventional fluid (400 ml/24 hr) intake. These increments in retained fluid were interpreted as arising from the preformed water and water of oxidation of catabolized tissue. From the same unit, Rush et al. observed delayed appearance of granulation tissue and wound healing in this syndrome and noted that "frequently the onset of diuresis and a fall in nonprotein nitrogen seemed to coincide with a sudden increase in granulation tissue and accelerated wound healing." The increased virulence of sepsis in these catabolic patients was noted as a major cause of death and it was postulated that there might be impairment of various host defense mechanisms in renal failure. Levenson, commenting on healing of battle wounds in face of severe renal dysfunction in these patients speculated, "It has been postulated by some that no attempt be made to reverse the "catabolic" reaction because it is a "defense mechanism" to supply metabolites to the injured area. There is not concrete evidence to support this." (In my preliminary studies muscle protein synthesis actually increased over first 48 hours during acute renal failure secondary to ischemia and rhabdomyolysis in contrast to results with ischemia alone where no change in protein synthesis observed, suggesting that products of muscle catabolism may have supplied substrates for synthesis). Levenson also studied the pattern of plasma amino acids in these patients early in their clinical course noting an increase in tyrosine and phenylalanine concentrations (which could serve as a precursor for hepatic gluconeogenesis). He also observed that the intensity of urinary nitrogen loss following injury may be decreased by the injection of testosterone proprionate and raised the following question, "Since the anabolic effects of testosterone are different for different tissues, what does the decrease in urinary nitrogen excretion mean in terms of wound healing?"

Although the catabolic state is recognized to be a major factor leading to mortality in this reversible disease process, little research has been directed

at those factors influencing negative muscle protein balance in acute renal failure. This is an area of special importance since muscle mass accounts for approximately 40% of the body tissue mass (6) comprises the major body protein reserve, is an active metabolic pool and contains significant quantities of electrolytes and water. The protein catabolism and survival in ARF may be modified by therapy has been suggested by a study in man in which it was reported that hyperalimentation with essential amino acids and glucose decreased morbidity and increased survival in a randomly selected group (7). In other studies, lower mortality rates have been reported following use of amino acid infusions (8), but these have been essentially uncontrolled and difficult to evaluate due to the wide range of "uncontrollable and often unrecognized variables inherent to seriously ill patients with this syndrome" (9). Nevertheless, these findings have stimulated many other studies in which the effect of amino acid therapy on overall survival, renal cellular repair, improvement in renal function in experimental acute renal failure has been explored. Results have been conflicting (9-13).

C. Experimental Design and Methods:
   1. Overall Experimental Approach:
      Utilizing well characterized in vitro methods, it is possible to measure net protein breakdown and protein synthesis simultaneously in the same isolated muscle, and from these calculate protein degradation. A unique feature of this system is that representative muscles (diaphragm, heart, extensor digitorum longus (EDL), soleus) obtained from the same animal can be incubated and studied in parallel in the same experiment. This approach facilitates the detection of different responses amongst various specialized muscle tissues, (11) which may be of importance in targeting therapy to a specific muscle, rather than furnishing net changes in the economy of the body muscle protein as a whole (12).

   2. Detailed Descriptions:
      a. Introduction of Acute Renal Failure
         Littermate male Sprague-Dawley rats, 80g (Charles River Laboratories) are utilized 4-5 days after receipt. Reproducible ischemic ARF is produced following pentobarbital anesthesia, by isolating and cross-clamping both renal pedicles for one hour. The surgical approach necessitates a one inch long mid-dorsal incision at the level of the kidneys, followed by renal exposure through a second set of incisions at the costovertebral angles. The kidneys are easily exposed and delivered by anterior abdominal pressure. They are decapsulated to preserve the adrenal glands and toothless alligator clamps applied for one hour across the vascular pedicles close to the hilum. The same procedure is followed in the sham-operated rats, save for the application of the alligator clamps. Muscles isolated from these rats were studied at 0, 24, 48, 72, and 96 hours post-operatively. The animals are permitted free access to water but no food during the 96 hours studied.

This initial protocol was selected so that the results, observed following nutritional deprivation, anesthesia and surgery could act as a baseline for com-

parison to those following the various experimental forms of nutritional supplementation. In any case, intestinal activity and food intake for 8-10 hours post-operatively was observed to be decreased in both groups.

b. Muscle Incubation Studies:
1) Theoretical Rationale:
The rate of protein synthesis is determined by measuring the rate of incorporation of ^{14}C-phenylalanine into muscle protein after correcting for intracellular specific activity (14). The rate of protein degradation is determined by measuring the net release of tyrosine from cell protein. Tyrosine is used as a measure of net protein degradation because this amino acid is neither synthesized nor catabolized by muscle. Consequently, its production by isolated muscles reflects net protein breakdown (15). The absolute rate of protein degradation is calculated as net protein breakdown plus the rate of protein synthesis.

2) Analytical Procedures:
Protein degradation and synthesis are measured using in vitro incubation techniques in isolated thin muscles, which allow rapid diffusion of substrates and gases, and which can be maintained for many hours in good physiological state (e.g., the red soleus muscle, the pale extensor digitorum longus, and cardiac auricular appendages (atrial strips) and the quarter diaphragm (16).

Rats with acute renal failure or controls are killed by cervical dislocation, the muscles rapidly removed and placed in Krebs-Ringer bicarbonate buffer (KRB), saturated with a 95% O_2 - 5% CO_2 gas mixture at pH 7.4. The tissues are rapidly rinsed, blotted, weighed on a torsion balance, and placed in sequence into 25 ml flasks containing 3 ml of gassed KRB at 37° with shaking. The muscles are then transferred to flasks containing 3 ml of fresh medium containing glucose, ^{14}C-labeled and non-radioactive phenylalanine (0.5 mM) with or without the specific substrate to be tested and incubated for two hours. The tissue is then removed from the medium, blotted, placed in 0.2M perchloric acid and once in 95% alcohol-ether (1:1), dissolved in soluene (Packard), and counted in a liquid scintillation spectrometer to assay for ^{14}C phenylalanine synthesis in the isoluable muscle protein fraction. The medium is covered for two minutes, centrifuged and the supernate analyzed for tyrosine, and alanine. Tyrosine is determined by the fluorometric method of Waalkes and Udenfriend (17) and alanine analyzed fluorometrically followed methods outlined in Bergmeyer (18).

3. Serial Studies:
Effect on body weight, serum urea nitrogen, creatinine, muscle wet weight, serum glucose and insulin levels in rats under study will be correlated with the above studies of muscle protein turnover.

4. Statistical Analysis:
The student "t-test" was employed for the statistical analysis of the data (19).

D. Results

   1. Effect on body weights (Figure 1).

      The body weights of both groups fell progressively at an identical rate through 72 hours. By 96 hours, the rats with ARF weighed significantly less than their sham-operated counterparts whose weights were unchanged from those at 72 hours.

   2. Effects on Serum Urea Nitrogen (SUN) and Creatinine (Figure 2).

      Both the SUN and creatinine concentrations rose abruptly within 24 hours following the induction of ARF. Although the SUN levels following ARF remained elevated over the 96 hours studied, the serum creatinine fell significantly after 48 hours yet remained higher than that of the shams. The creatinine urine/plasma (u/p) ratios following ARF remained low (12.7-19.1), compared to the shams, whose values ranged from 64.9-67.5, suggesting continuation of poor renal function during this period, despite the fall in serum creatinine concentrations. In the sham-operated group, the SUN levels rose slowly and significantly every 24 hours by approximately 7 mg/dl, most likely reflecting starvation and endogenous tissue breakdown, whereas concomitantly, the serum cretanine concentrations remained stable.

   3. Muscle Wet Weights (Figure 3).

      Since both the soleus and EDL were isolated intact at the tendons, their total weights could be accurately measured. Form "0" time through the first 48 hours, soleus muscle weights in both the sham and ARF groups remained similar and were unchanged. A break occurred by 72 hours at which time there was a significant but identical fall in muscle weights in both groups. Thereafter the soleus weights from the sham-operated rats remained constant through 96 hours while those from rats with ARF continued to fall significantly from 72 to 96 hours.

      The weight of the EDL muscles from the sham-operated rats did not change during the first 24 hours. Thereafter, there was a significant fall through 48 and 72 hours with no further change to 96 hours. The weight of the EDL muscles from the ARF group fell significantly and progressively every 24 hours sthrough 96 hours and except for 72 hours were consistently lower than the EDL weight of the sham operated group.

   4. Measurement of Muscle Protein Turnover.

      As outlined, simultaneous measurements were made of muscle protein synthesis and net protein breakdown every 24 hours from 0 through 96 hours post operatively. As already noted, protein degradation was calculated as the algebraic sum of the two and expressed as tyrosine released in nm/mg weight tissue/2h. Likewise, protein synthesis is expressed as nm of tyrosine incorporation per mg wet tissue 2 h. To test the effect of a key branch-chain amino acid known to reduce muscle protein degradation and increase protein synthesis, 1-leucine, 0.85 mM (5 x the normal rat plasma level) was added to the muscles in one half of the incubation vessels. For comparison no leucine was added to the media bathing the other of the paired muscles. For purposes of simplicity, the
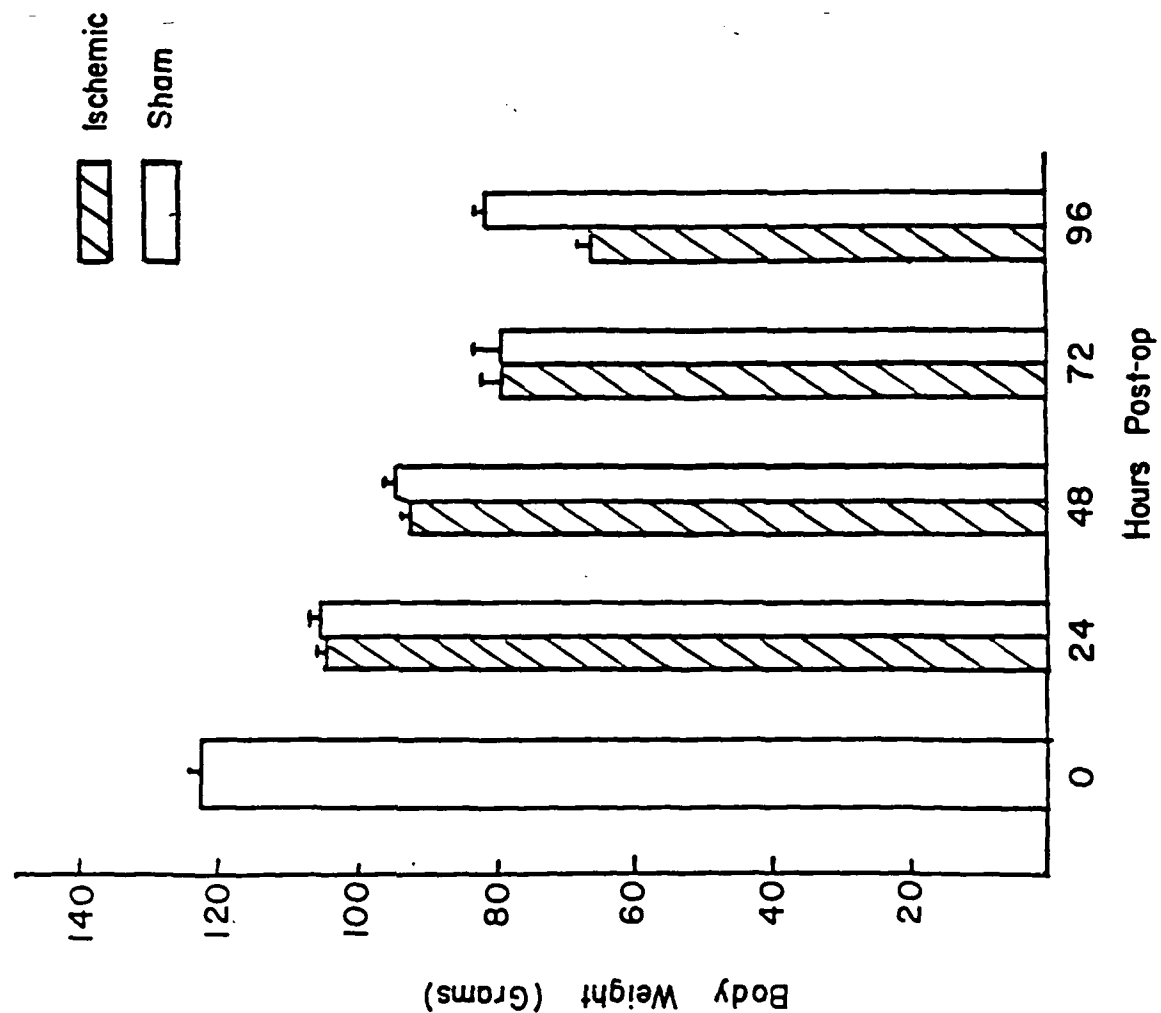
Figure 1

RAT WEIGHTS

Figure 2

SERUM CREATININE

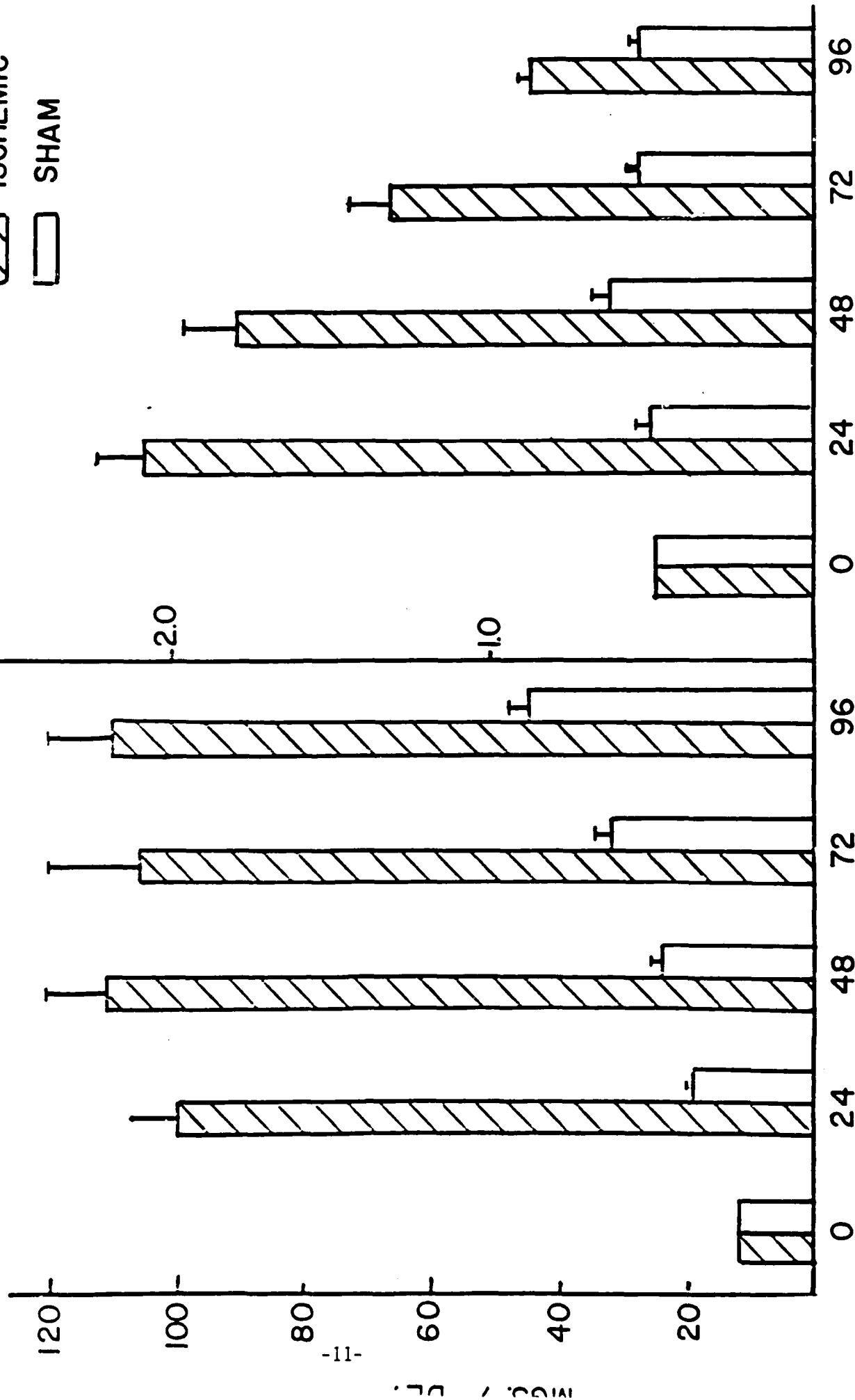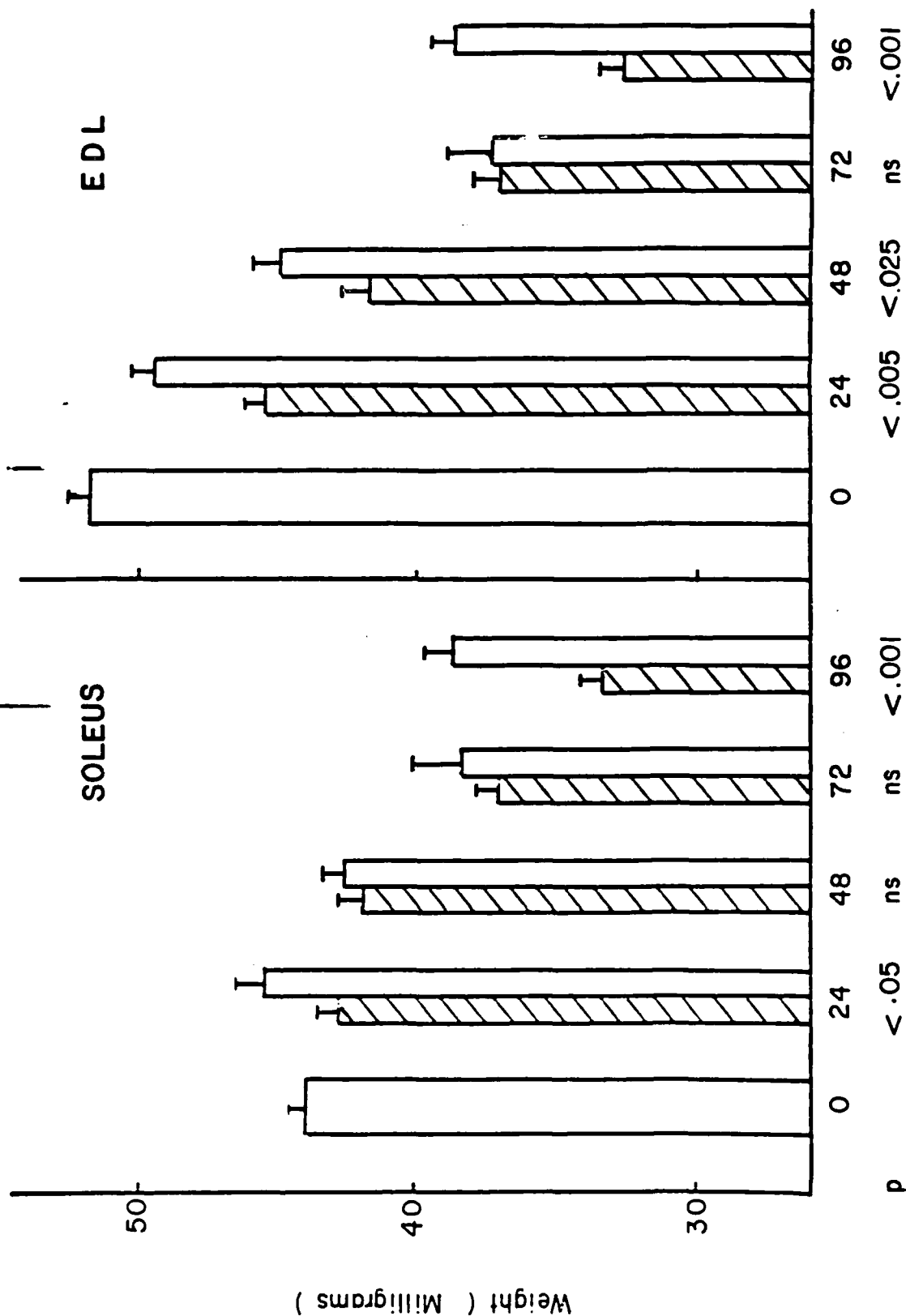SERUM UREA NITROGEN

ISCHEMIC
SHAM

-11-

Figure 3

MUSCLE WET WEIGHT

Figures depict the experiments in which the leucine only was included in the media. The complete data with and without the leucine media supplementation is included in the Tables.

a. Diaphragm (see Figure 4 and Table I):
Within the first 24h post-operatively, protein degradation (PD) in the diaphragm from both the ischemic and sham-operated groups rose significantly (p <0.025), (an increase unaffected by media leucine) which persisted through the 96 hours of study. Concomitantly, irrespective of the presence of media leucine, protein synthesis (PS) in diaphragms from rats with ARF remained unchanged from baseline levels for 24 hours. PS then fell significantly by 48 hours (p < 0.025) and continued to decrease slowly thereafter. In the sham operated group, again irrespective of the presence of media leucine, PS fell stepwise and significantly from baseline levels at both 24 hours and 48 hours and continued at low levels of activity through 96 hours.

b. Heart (See Figure 5 and Table II)
In sharp contrast to the findings in diaphragm, media leucine enhanced both protein synthsesis and degradation in atrial muscle. Thus, in ARF, in the presence of media leucine, protein degradation remained relatively stable at baseline levels for at least 72 hours, then rose abruptly. Without leucine, PD in the same tissue was significantly elevated at) (p < 0.01), 24 (p <0.05), and 48 (p < 0.001) post-operatively. In the sham-operated group, a dif-ferent ARF at 24 h (p < 0.005), falling sharply to baseline levels by 48h. Again without media leucine, PD became significantly elevated in atrial tissue at 0, 24 and 48h post-operatively in the sham-operated group.

Protein synthesis remained at baseline levels for at least 48 hours in ARF in the presence of leucine, then plunged from 48 hours to 96 hours. In the sham-operated group, stability of synthesis with media leucine persisted only

c. Soleus (See Figure 6, Table III):
Twenty-four hours post ARF, in the presence of media leucine, PD in the soleus muscle rose significantly above baseline levels (p < 0.025) and sharply above sham-operated levels (p < 0.001). Thereafter, PD in ARF fell to baseline levels at 48 hours, persisted there for 72 hours and sharply rose by 96 hours. In the sham-operated rats, soleus PD rose from 24 to 48 hours (p <0.025) and remained stable. In both level cases without media leucine supplementation, synthesis would have been significantly decreased during this period.

d. Extensor Digitorum Longus (Figure 7, Table IV):
Irrespective of media leucine, PD in the EDL from rats with ARF rose significantly by 24 hours (p >0.001) and also above sham-operated levels (p< 0.005). Thereafter, PD gradually and progressively increased in both groups through 96hours. Concomitantly, protein synthesis in both groups fell progressively and significantly over the first 48 hours post-operatively, then remained stable at a low level of activity through 96 hours. When leucine was absent from the media, synthesis was lower in both groups.

Figure 4
DIAPHRAGM

PROTEIN SYNTHESIS

PROTEIN DEGRADATION

nm Tyrosine / mg tissue / 2 hrs

Hours Post-op

- - - - Ischemia
——— Sham
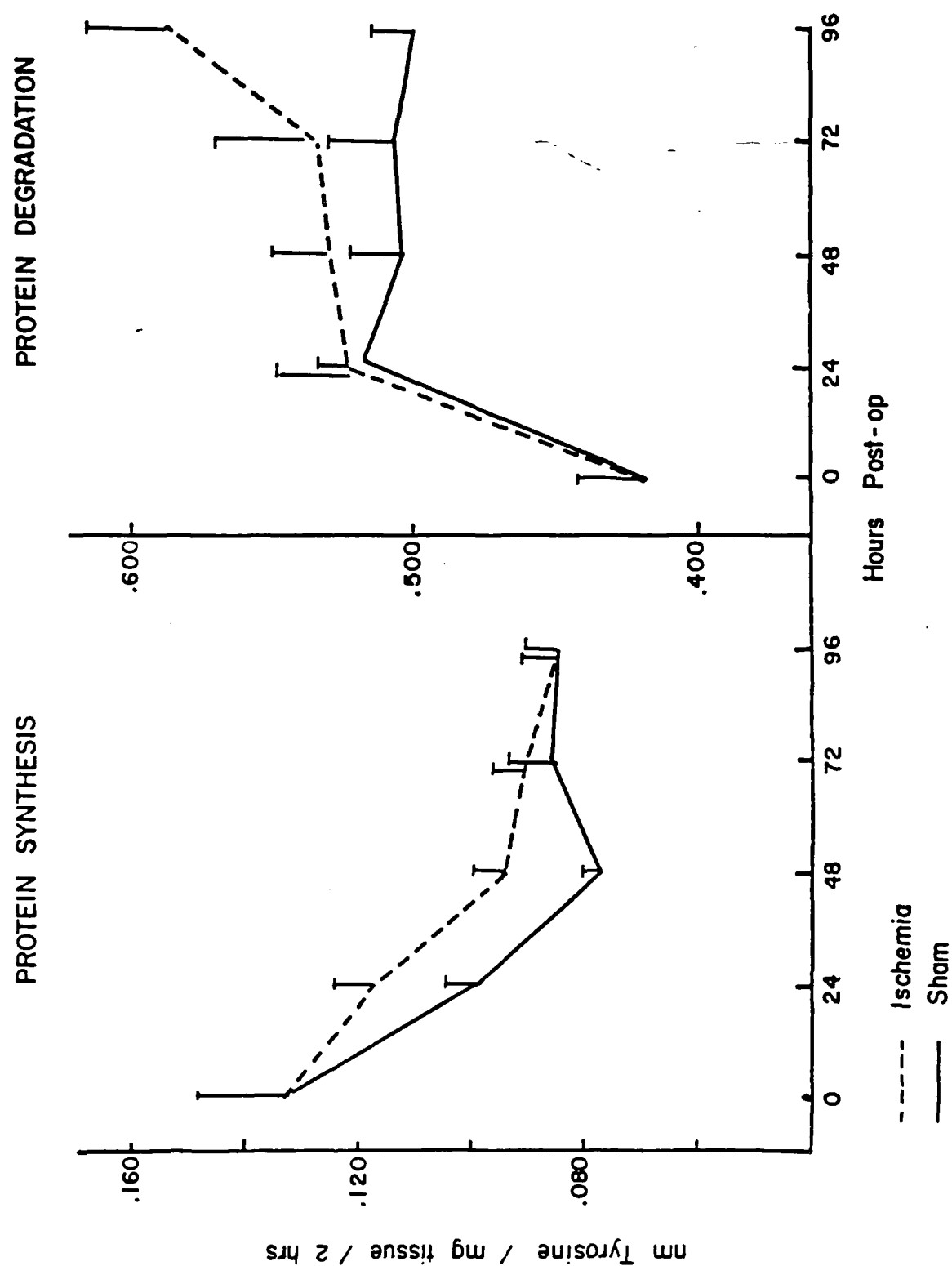
TABLE 1

DIAPHRAGM

Protein Degradation

nm Tyrosine released/mg tissue/2h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	– leucine	+ leucine	– leucine
0	0.419±023 (7)	0.523±.027 (8)	0.419±.023 (7)	0.523±027 (8)
24	0.523±.024 (18)	0.497±.043 (6)	0.519±.015 (18)	0.526±.020 (6)
48	0.530±.019 (16)	0.605±.031 (6)	0.504±.017 (17)	0.498±.015 (5)
72	0.534±.035 (16)	0.642±.030 (5)	0.507±/023 (9)	0.700±.029 (3)
96	0.588±.027 (12)	0.587±.017 (5)	0.501±.013 (14)	0.571±.034 (3)

Protein Synthesis

nm Tyrosine incorporated/mg tissue/2 h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	– leucine	+ leucine	– leucine
0	0.133±.014 (7)	0.115±.006 (8)	0.133±.014 (7)	0.115±.006 (8)
24	0.116±.007 (18)	0.118±.011 (6)	0.098±.006 (18)	0.093±.005 (6)
48	0.094±.005 (16)	0.066±.007 (8)	0.077±.003 (17)	0.064±.002 (5)
72	0.091±.005 (16)	0.071±.C08 (5)	0.086±.007 (9)	0.063±.003 (3)
96	0.086±.005 (12)	0.062±.004 (5)	0.085±.005 (14)	0.081±.006 (3)

Values are expressed in terms of means ± one standard error.
Numbers in parentheses below indicate number of muscles incubated.

Figure 5

HEART

PROTEIN SYNTHESIS

PROTEIN DEGRADATION

nm Tyrosine / mg tissue / 2 hrs

Hours Post-op

----- Ischemia

—— Sham

-16-

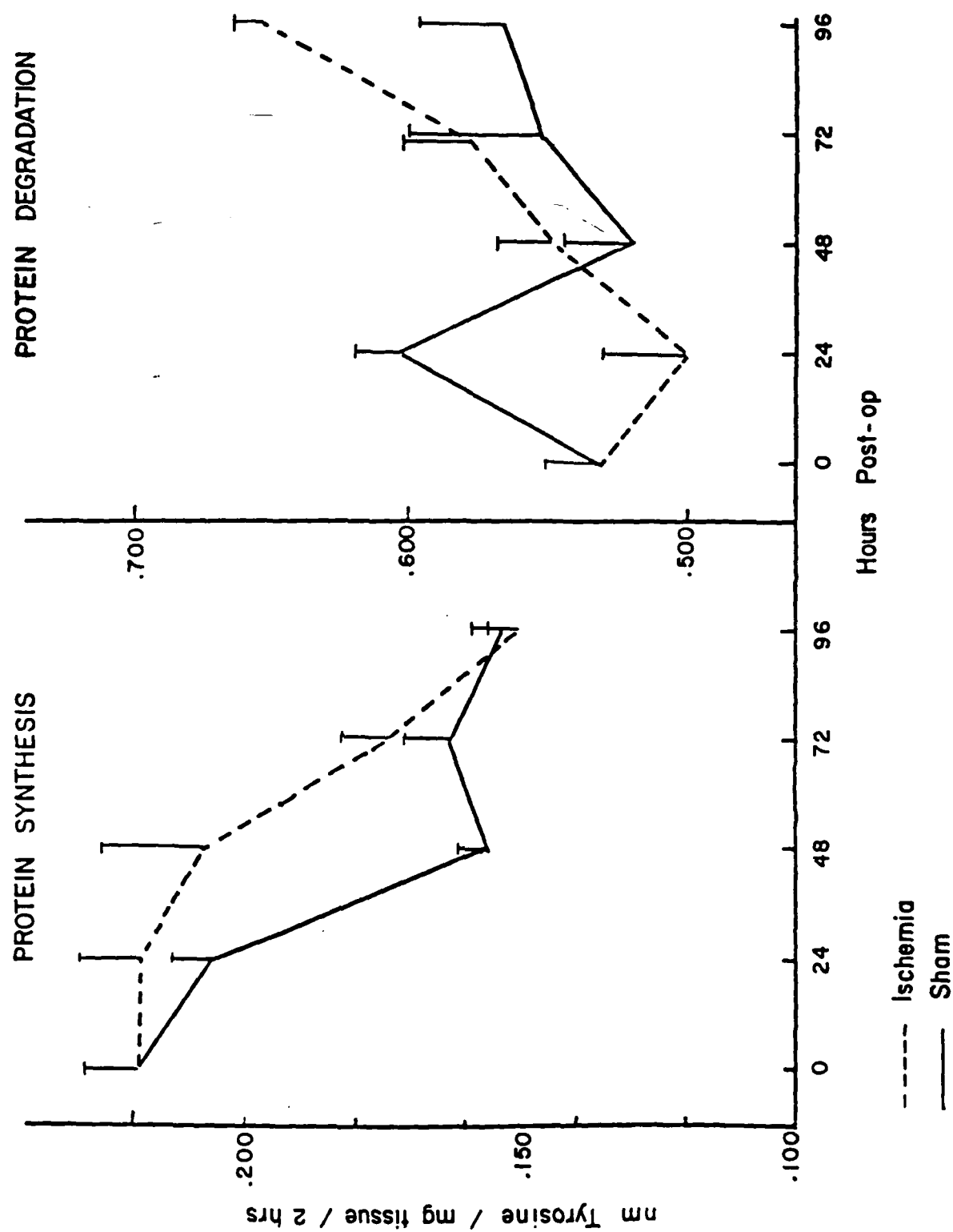# TABLE II

ATRIA

Protein Degradation

nm Tyrosine released/mg tissue/2 h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	− leucine	+ leucine	− leucine
0	0.531±.02 (7)	0.619±.026 (8)	0.530±.02 (7)	0.619±.026 (8)
24	0.500±.03 (11)	0.594±.022 (11)	0.603±.07 (20)	0.795±.027 (6)
48	0.548±.02 (8)	0.772±.05 (7)	0.519±.024 (12)	0.779±.031 (6)
72	0.579±.023 (12)	0.581±.065 (5)	0.552±.048 (5)	0.659±.048 (3)
96	0.652±.011 (7)	0.713±.071 (5)	0.565±.031 (11)	0.799±.006 (3)

Protein Synthesis

nm Tyrosine incorporated/mg tissue/2 h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	− leucine	+ leucine	− leucine
0	0.219±.01 (7)	0.210±.01 (8)	0.219±.01 (7)	0.210±.01 (8)
24	0.219±.01 (11)	0.217±.007 (12)	0.206±.007 (21)	0.215±.006 (6)
48	0.208±.018 (8)	0.152±.009 (7)	0.156±.005 (12)	0.167±.009 (5)
72	0.174±.008 (12)	0.104±.009 (5)	0.163±.009 (5)	0.136±.016 (3)
96	0.150±.006 (7)	0.126±.010 (5)	0.153±.006 (10)	0.176±.013 (3)

Values given in terms of means ± one standard error.
Numbers below indicate the number of paired atria incubated, i.e. two atria per flask.

-17-

Figure 6

SOLEUS

PROTEIN DEGRADATION

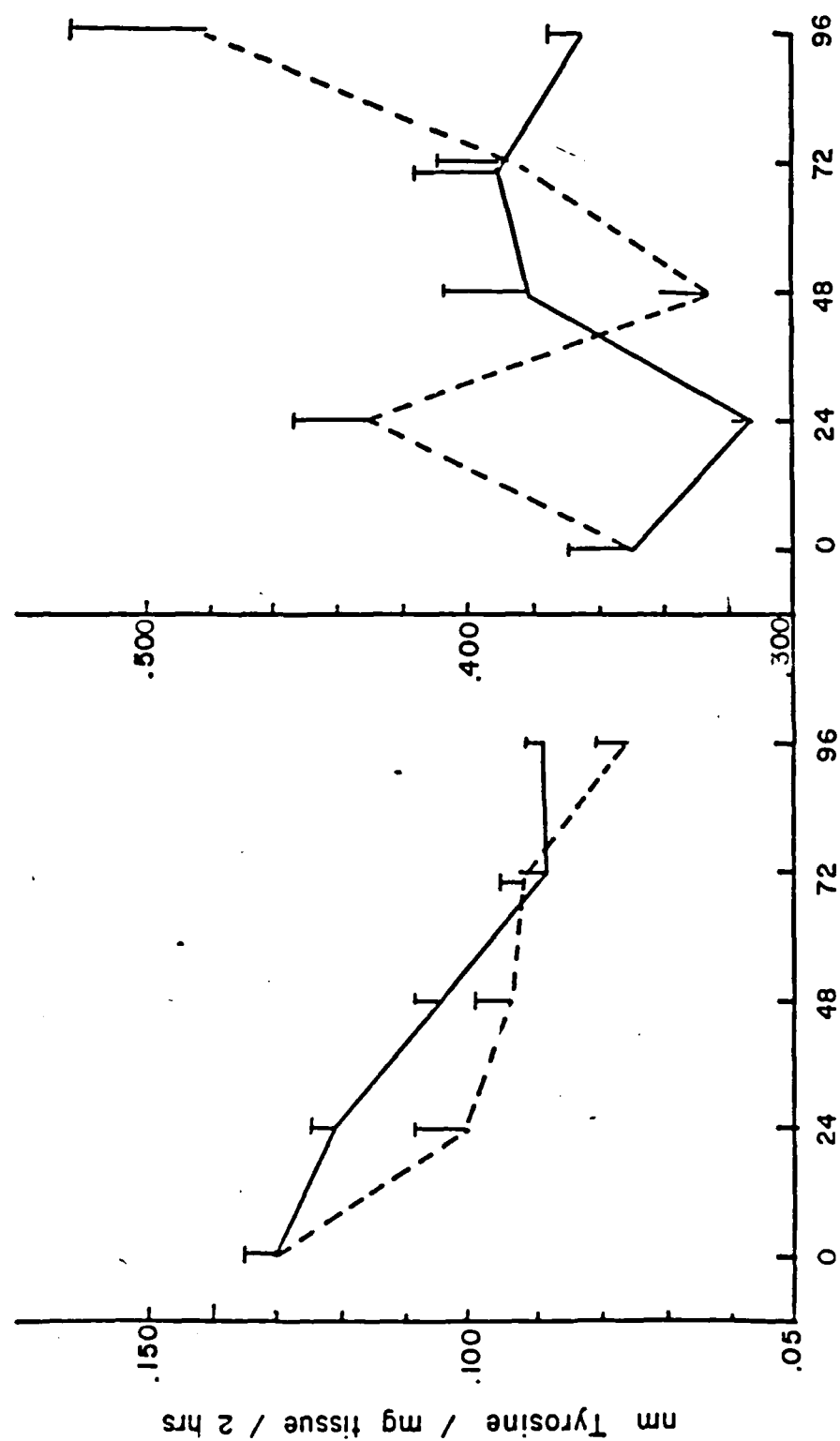PROTEIN SYNTHESIS

nm Tyrosine / mg tissue / 2 hrs

TABLE III

SOLEUS

Protein Degradation

nm Tyrosine rleased/mg tissue/2 h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	– leucine	+ leucine	– leucine
0	0.350±.021 (12)	0.416±.021 (12)	0.350±.021 (13)	0.410±.021 (12)
24	0.429±.023 (10)	0.428±.020 (13)	0.312±.008 (15)	0.347±.012 (15)
48	0.326±.016 (12)	0.379±.017 (13)	0.382±.026 (9)	0.374±.01 (8)
72	0.384±.026 (27)	0.584±.09 (5)	0.391±.026 (14)	0.389±.017 (3)
96	0.485±.038 (17)	0.735±.033 (5)	0.365±.012 (23)	0.463±.028 (3)

Protein Synthesis

nm Tyrosine incorporated/mg tissue/2 h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	– leucine	+ leucine	– leucine
0	0.130±.005 (14)	0.127±.007 (12)	0.130±.005 (14)	0.127±.007 (12)
24	0.101±.008 (12)	0.067±.005 (13)	0.121±.004 (15)	0.080±.007 (15)
48	0.094±.006 (14)	0.069±.004 (14)	0.104±.005 (9)	0.780±.007 (10)
72	0.076±.005 (19)	0.044±.002 (5)	0.089±.003 (24)	0.067±.003 (3)
96	0.092±.004 (27)	0.057±.006 (5)	0.088±.005 (15)	0.073±.008 (3)

Values given in terms of means ± one standard error.
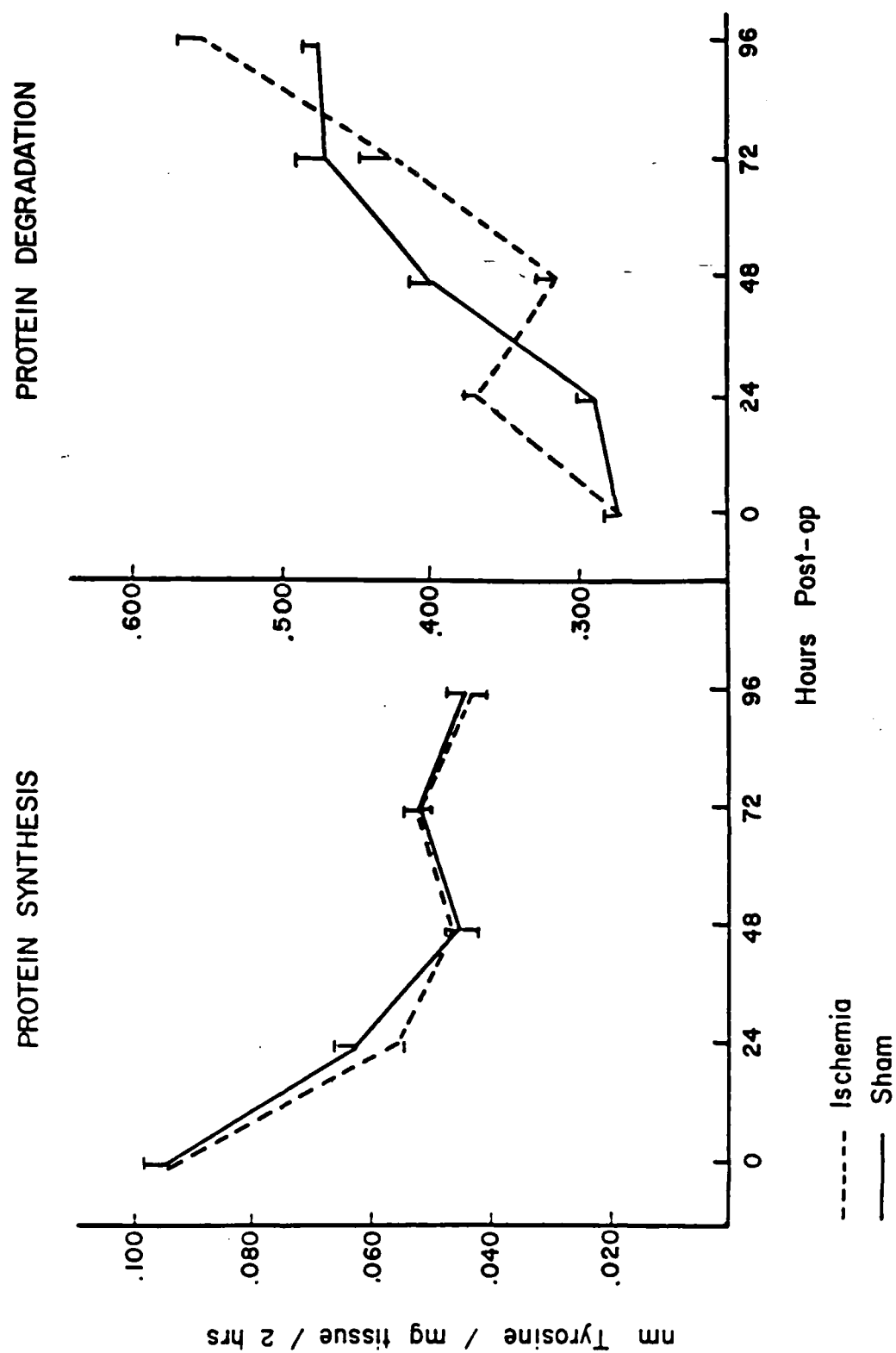Numbers in parentheses below indicate number of individual muscles incubated.

Figure 7
E.D.L.

PROTEIN DEGRADATION

PROTEIN SYNTHESIS

Hours Post-op

nm Tyrosine / mg tissue / 2 hrs

----- Ischemia
—— Sham

TABLE IV

EXTENSOR DIGITORUM LONGUS

Protein Degradation

nm Tyrosine released/mg tissue/2 h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	– leucine	+ leucine	– leucine
0	0.268±.014 (14)	0.297±.008 (15)	0.268±.014 (14)	0.297±.008 (15)
24	0.365±.013 (11)	0.388±.02 (11)	0.286±.014 (8)	0.266±.01 (9)
48	0.313±.016 (14)	0.346±.017 (15)	0.399±.017 (10)	0.445±.02 (10)
72	0.421±.026 (29)	0.639±.04 (5)	0.468±.02 (15)	0.513±.023 (3)
96	0.552±.018 (18)	0.674±.023 (5)	0.472±.015 (25)	0.573±.029 (3)

Protein Degradation

nm Tyrosine incorporated/mg tissue/2 h

Group	Acute Renal Failure		Sham-Operated	
Media	+ leucine	– leucine	+ leucine	– leucine
0	0.093±.004 (14)	0.080±.004 (15)	0.093±.004 (14)	0.080±.004 (15)
24	0.053±.002 (12)	0.043±.002 (14)	0.062±.004 (9)	0.053±.003 (10)
48	0.046±.002 (14)	0.038±.002 (16)	0.044±.003 (10)	0.036±.002 (10)
72	0.052±.002 (29)	0.036±.008 (5)	0.052±.003 (15)	0.039±.006 (3)
96	0.042±.002 (19)	0.033±.002 (5)	0.045±.002 (25)	0.045±.002 (3)

Values are expressed as means ± one standard error.
Numbers in Parentheses below indicate number of muscles studied.

Figure 8

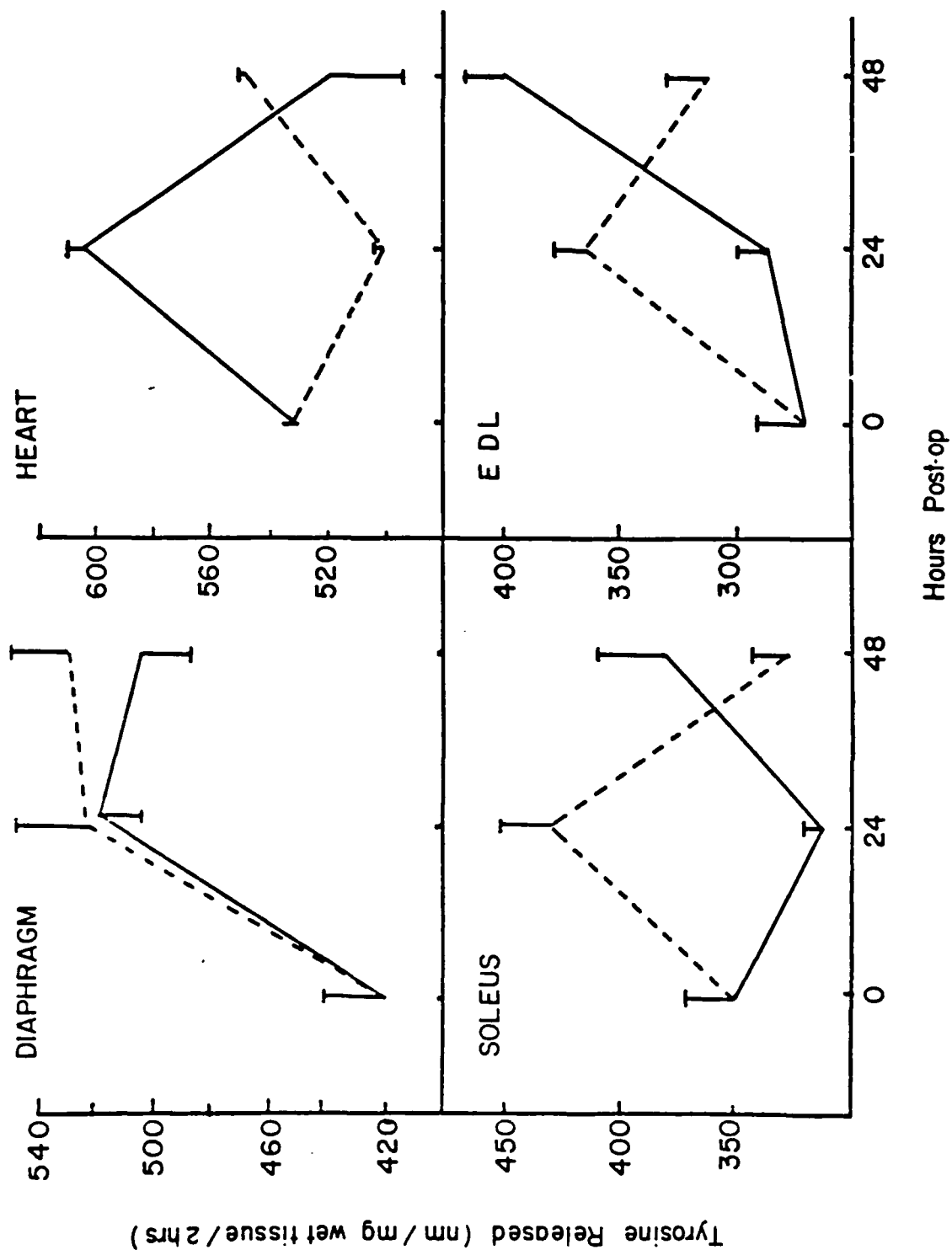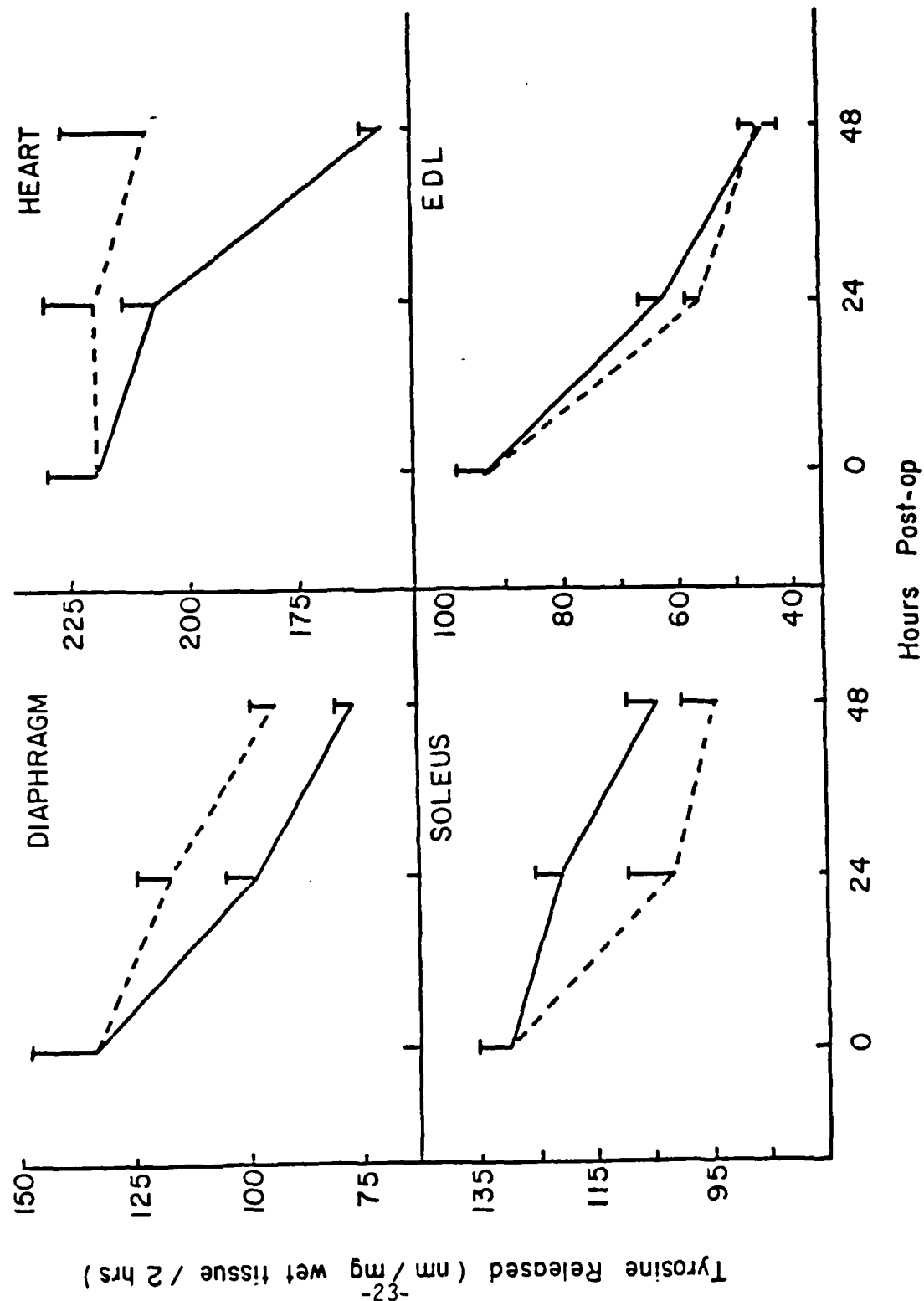# MUSCLE PROTEIN DEGRADATION

----- Ischemic
——— Sham

HEART

DIAPHRAGM

E D L

SOLEUS

Hours Post-op

Tyrosine Released (nm / mg wet tissue / 2 hrs)

Figure 9

MUSCLE PROTEIN SYNTHESIS

Summary of Results

In this model of <u>acute</u> <u>renal</u> <u>failure</u> and nutritional deprivation, the following generalizations can be made on the overall pattern of protein turnover in these four muscles:

1. Acute renal failure strikingly influenced the pattern of muscle protein degradation in the four muscles studied during the first 24 hours of this insult (Figure 8). The effect of ARF differed among the muscle groups. ARF increased protein degradation in the soleus and EDL, compared to controls whereas in the heart, the opposite obtained. No difference between experimental and control values were noted in diaphragm.

2. The overall pattern of protein synthesis in all muscles appeared similar, i.e., a general decline in synthetic activity over time irrespective of whether the initial insult was ARF or the control procedure (Figure 9).

3. There was no necessary relationship linking synthesis to degradation amongst the various muscles.

4. As a result, muscle wasting and negative nitrogen balance occurred in both groups of rats. Both the soleus and EDL, evidenced weight loss over time, albeit with characteristically <u>different</u> patterns. The weight of the soleus appeared more stable for at least 48 hours. The EDL sustained weight loss almost within the first 24 hours post-operatively, that from the rats with ARF were affected the most.

5. Increasing levels of the serum urea nitrogen were observed in the sham operated rats in the face of a stable serum creatinine, while in the rats with ARF, high SUN concentrations persisted despite falling serum creatinine levels after 48 hours.

6. The presence of leucine in the media either enhanced synthesis or decreased degradation in some but not all muscles. Notably, protein degradation was markedly diminished by media leucine in the heart over the first 72 hours, indicating targeting of a specific important muscle by a specific nutriment in the media.

Comments and Pitfalls of These Results:

Two complex processes are occurring in this experimental setting in the rat as in human casualties, who by their injuries are unable to ingest orally: acute renal failure and progressive starvation. The effects of ARF per se are best seen in the first 24 hours, although the effect of food deprivation on these results obviously cannot be completely eliminated. The rat is a nocturnal feeder. The operative procedures were performed between 8:30 and 9:30 AM at which time the stomachs and intestines were still full of food. Post-operatively, the stomachs and intestines were partiallyyempty by 24 hours. By 48 hours, the stomach and small intestine were completely empty, the intestinal

-24-

contents were confined to the large colon, however fat stores appeared depleted. In a study in which the same in vitro incubation techniques were utilized as in this report, Li and Goldberg demonstrated that after 24 hours of "simple" food deprivation, protein synthesis decreased in the EDL but not invariably in the soleus. Concomitant protein degradation in these two muscles did not change during the first 24 hours; a significant increase was observed only after 48 hours. Reference to Figure 8 in which muscle protein degradation is plotted through the first 48 hours of study in all four muscles in rats with ARF or in shams, it is demonstrated that the pattern of protein degradation varied from muscle to muscle at 24 hours, a situation significantly different from the predominating pattern of starvation which followed. Thus, in this model, it seems reasonable to assume that the predominant stress within the first 24 hours was that of acute renal failure, starvation becoming the more important factor thereafter. Figure 9 demonstrates the pattern of protein synthesis in the four muscles during the first 48 hours.

The question may be raised as to whether it might be more accurate to express results in terms of "dry weight" or "mg of protein" as changes in hydration may occur and there may be loss of muscle glycogen and fat over time. Quantitation of these parameters are in the process of being performed. Preliminary results suggest that no significant differences between those obtained using wet tissue weights have occurred. In a study of food deprivation lasting for 48 hours (employing male rats with an initial weight of 80g), Li and Goldberg observed that the protein concentration of the EDL and soleus increased only slightly, suggesting a selective loss of cellular non-protein components (glycogen, fat, RNA). In these same muscles, the dry weight-to wet weight ratios did not change during the 48 hour fast, the dry weight decreased in parallel with the wet weight. In the same study using larger rats averaging 140g at onset and starved for 5 days, Li and Goldberg reported a 32% loss of carcass weight. The heart, diaphragm, plantaris, tibialis anterior, extensor digitorum longus decreased in wet weight roughly proportionally (28-32%), whereas the wet weight of the soleus did not fall significantly.

Further Results:
Effect on Serum Glucose and Insulin:
a. The pattern of insulin secretion and concomitant blood sugar concentrations are of interest in this model of trauma and food deprivation since within 24-48 hours, bodily glycogen stores are largely depleted, fat deposits grossly shruinken and gluconeogenesis from muscle protein a principal source for blood glucose maintenance.

We were very fortunate to have the assistance of Dr. David Robbins of the Metabolic Unit of the Department of Medicine here at the University of Vermont, who performed serum immunoreactive insulin levels utilizing specific rat insulin antibodies (Figure 10). From these serial studies, it seems clear that irrespective of food deprivation, the serum glucose levels are held within normal limits over the 96 hours studied. Moreover, this stability in serum glucose homeostasis exists irrespective of wide variations in serum immunoreactive insu-

lin concentrations noted especially during the first 24 h post ARF. Further studies are projected on insulin levels and insulin receptor function, especially during the first 24 hours post-induction of ARF.

These results were presented at the National Meetings of the American Federation for Clinical Research in Washington, DC, in 1982.

With the first phase of this study completed, we have had the benefit of a thorough critical review of our findings by Dr. Robert Low, Professor of Physiology at the University of Vermont, whose main scientific interest is in muscle protein turnover. Dr. Low suggested that another set of controls was necessary to complete this in vitro phase of our work. This includes rats inawhicheacute renal failure is induced by bilateral renal ischemia but having free access to food (as Purina Rat Chow) and water and a group of sham-operated rats also with free access to rat chow. We plan to follow his suggestions and submit the results of our studies for publication in Kidney International.

Figure 10

EFFECT OF ARF AND NUTRITIONAL DEPRIVATION ON SERUM GLUCOSE AND INSULIN
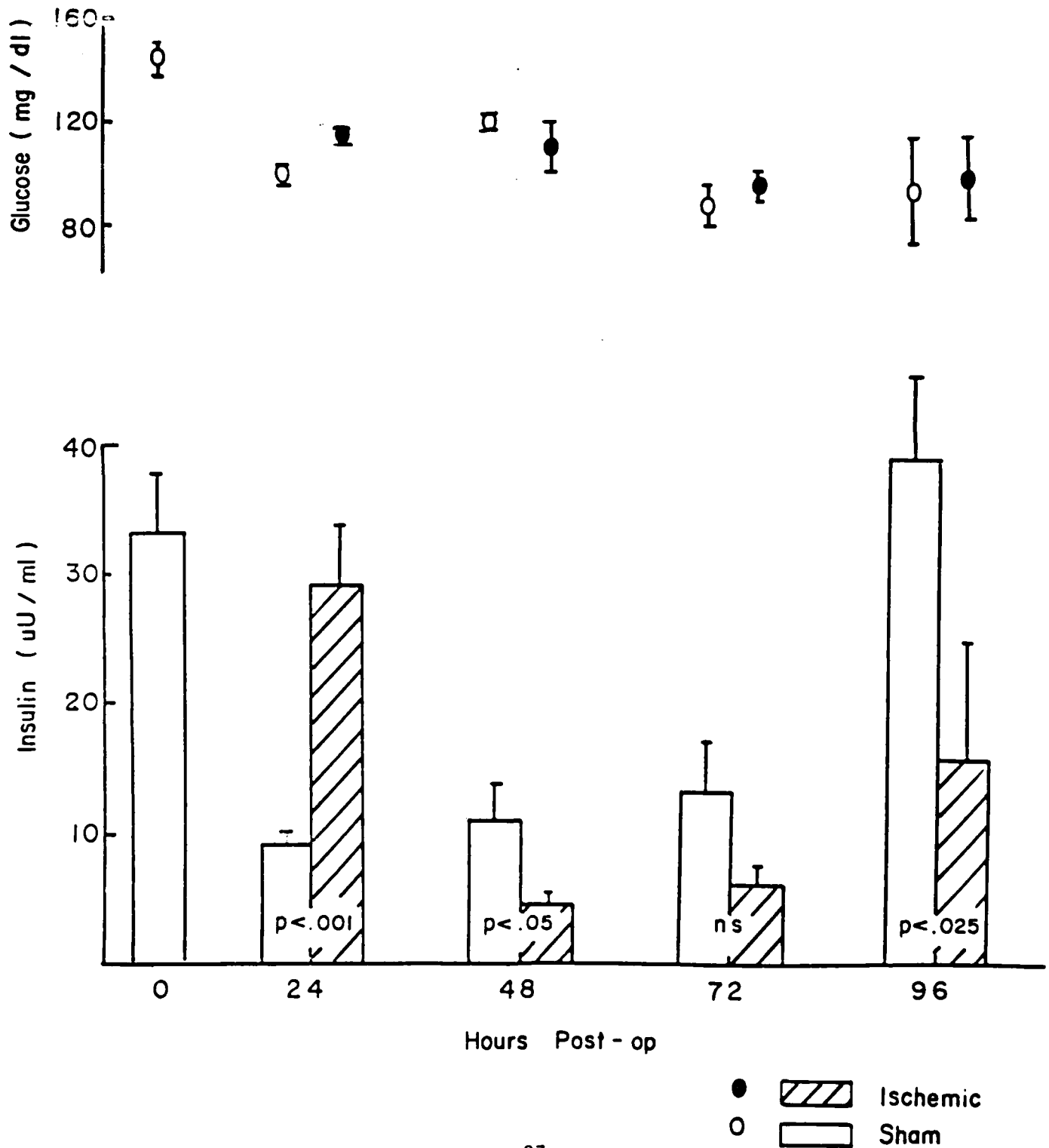
In Vivo Infusion Studies

A. Introduction

With the near completion of the in vitro studies described above, the next phase of this study was approached. The goal of this phase was to test the in vivo efficacy of those substrates demonstrated in vitro to be effective in reducing muscle protein turnover in experimental acute renal failure. Our overall plan was to induce ARF and intravenously infuse rats with a series of potentially therapeutic solutions for 24 or 48 hours, sacrifice them, remove and incubate their muscles in vitro and measure protein turnover using our standard methods. For our initial studies, we decided to test the relative efficacy of the following:

a) Hypertonic glucose (23%) alone versus hypertonic glucose plus insulin

b) Hypertonic glucose plus leucine (0.85 mM) versus hypertonic glucose plus a commercial mixture of essential and non-essential amino acids. (Parenthetically, it is of interest that these solutions are relatively commonly used in humans with acute renal failure).

To perform these experiments, a number of technical questions had to be resolved:

1. Was it practical to routinely and rapidly catheterize the jugular vein of 6-8 anesthetized small rats at "one sitting," in rats that had just had ischemic ARF induced?

2. Was it better to contain the rats in small holders permitting only minimal muscular movement during the infusions, or was it preferable to allow complete freedom of movement in individual cages during the 24 hr infusions? What special equipment was required for each approach?

3. What is the total volume of fluid required to infuse 80g rats over a 24 hr period to compensate for possible "third space" losses following anesthetic, surgery and induction of ARF, avoiding both under and overhydration of these partially oliguric rats?

4. How much insulin should be infused together with hypertonic glucose so that a high level of serum insulin is reached and hypoglycemia avoided?

The satisfactory resolution of these problems took a great deal of time, trial and error, and advice from other investigators experienced in small animal techniques.

To summarize, we now routinely catheterize the jugular vein of 8-10 rats using flexible Tygon Microbore Tubing (internal diameter 0.015, external diameter 0.03 mm). Using a Harvard infusion pump withour own modifications, we can simultaneously infuse 10 rats intravenously over a wide series of rates, set as desired.

With the help of the Instrumentation Facility of the University of Vermont, an apparatus has been constructed which will gently, but separately, hold ten small (80-120 g) rats. Under each rat is a collecting system constructed to catch urine, separate from feces. A small opening in the top of each restraining device will admit catheters leading to the neck of the rat inside.

We have also devised a simple inexpensive harness which allows single rats with venous and arterial catheters inserted, to move freely about in their individual cages. These implanted catheters are protected from being chewed or torn as they are covered bvy a thin, flexible light-weight, closely coiled stainless steel spring. Fluids can be infused intravenously at a regular rate via the modified Harvard infusion pumps described above. The advantage of this second system is that muscle protein loss secondary to enforced immobilization can be prevented and comparisons in protein turnover can be made between post-operative rats rendered immobile or allowed free movement, if desired.

The volume of intravenous fluid required over the first 24 hours post induction of acute renal failure in 80 g rats is approximately 14 ml. Somewhat less is required for the second 24 h of infusion.

To determine the amount of insulin to be added to an IV infusion of hypertonic glucose, we employed the concept of an euglycemic clamp (studied by DeFronzo of Yale). We determined that regular insulin, infused at a rate of 10 milliunits/min/kg body weight, along with 23% glucose would give the desired results. We found at the end of a 24 h infusion of this combined solution, that serum glucose concentrations were between 150-180 mg/dl and serum insulin levels were 4=5 times higher than those normally circulating from endogenous sources.

Results:
    Our preliminarv findings clearly demonstrated that there were no significant differences in either muscle protein synthesis or in muscle protein degradation when the results using insulin plus hypertonic glucose were compared with those in which only hypertonic glucose were infused in littermates. Thus, at least during the first 24 hours post acute renal failure, the addition of insulin to hypertonic glucose does not facilitate an improvement in muscle protein balance.

Similarly, when we compared the effect of infusing a solution of hypertonic glucose containing all the essential and non-essential amino acids on muscle protein turnover with the results obtained using hypertonic glucose plus 0.85 mM leucine, we found no significant differences (See Table V). Currently, we are exploring the comparative effects of infusing these two solutions for 48 hours.

Discussion:
    Our tentative conclusions are that at least for the first 24 hours following induction of acute renal failure, no apparent improvement results in muscle protein balance irrespective of whether one uses hypertonic glucose with or without insulin, hypertonic glucose plus the essential and non-essential amino acids or hypertonic glucose plus 0.85 mM leucine alone.

TABLE V

The Effect of an IV Infusion of Hypertonic Glucose
Containing Either Balanced Amino Acids or Leucine (0.85mM)
on Muscle Protein Synthesis or Degradation
(1st 24 h following Acute Renal Failure)

	Protein Synthesis		Protein Degradation	
	+Amino Acids	+Leucine	+Amino Acids	+Leucine
		nmols/mg wet tissue/2 h		
Extensor Digitorum Longus	0.072+.008 (10)	0.073+.01 (6)	0.33+.02 (10)	0.31+.03 (6)
Soleus	0.112+.01 (7)	0.119+.01 (6)	0.396+.02 (9)	0.376+.02 (6)
Diaphragm	0.101+.005 (10)	0.110+.008 (6)	0.479+.01 (10)	0.488+.03 (6)
Heart	0.169+.02 (5)	0.156+.01 (3)	0.433+.04 (5)	0.470+.04 (3)

Values expressed as means ± one SEM. Numbers in parenthesis indicate no. of separate muscles studied.

These observations seem in accord with some recently published results which appeared after these studies were performed:

1) Baracose, V., Rodemann, H.P., Dinarello, C. and Goldbert, A.L. Stimulation of Muscle Protein Degradation and Prostaglandin E_2 Release by Leukocyte Pyrogen (interleukin-1). N. Eng. J. Med 1983, 308: 533-8.

2) Clowes, G.H., George, B.C., Villee, C.A., Jr., and Sarauls, C. Muscle Proteolysis Induced by a Circulating Peptide in Patients with Sepsis or Trauma. N. Eng. J. Med. 1983; 308: 545-52.

From these studies, it seems highly possible that there may be a group of circulating proteases induced by trauma and/or sepsis which result in muscle protein breakdown. We are currently exploring, in addition to pursuing our main line of research, the relationship of ribonuclease circulating in serum and in muscle in rats with acute renal failure. This enzyme has been implicated in several of the muscular dystrophies and has been found to be high in patients with chronic renal failure.

To summarize our findings to date: it seems clear (in our experimental setting) that the intravenous administration of hypertonic glucose alone following the induction of acute renal fialure is obviously superior to complete nutritional ceprivation and is as effective in maintaining protein balance as any of the so-called supplemental "super-therapeutic" acute renal failure concoctions. These results if further substantiated, would suggest that clinically the less expensive, more readily obtained, easily transported, stored and reconstituted solutions of hypertonic glucose should be utilized in acute renal failure.

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